

Prolyl 3-Hydroxylase and 4-Hydroxylase Activities in Certain Rat and Chick-Embryo Tissues and Age-Related Changes in their Activities in the Rat

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Prolyl 3-hydroxylase activity, expressed per unit of extract protein, was much higher in rat kidney cortex than in the lung, liver or skin. A marked decrease in activity was found in the kidney cortex, liver and skin beyond 10 days of age. The ratio of prolyl 3-hydroxylase to 4-hydroxylase activity in the kidney cortex was 13–17 times that in the skin, that in the liver 6–8 times, and that in the lung about twice the value for the skin, there being no changes in this ratio with age. In 16-day chick embryos the highest ratios of prolyl 3-hydroxylase to 4-hydroxylase activity were found in the liver, heart, lens, aorta and kidney, and the lowest ratios in tendon, cartilage, cartilaginous and membranous bone and skin. The results suggest that the differences in the extent of prolyl 3-hydroxylation between various collagens can in part be explained by differences in the amount of prolyl 3-hydroxylase activity among different cells.

Hydroxyproline is found in vertebrate tissues almost exclusively in collagen. Most of this imino acid is present in the form of the *trans*-4 isomer, but all collagens also contain some *trans*-3-hydroxyproline (for reviews see Cardinale & Udenfriend, 1974; Fietzek & Kühn, 1976; Piez, 1976). The 3-hydroxyproline content of type-I collagen from skin, tendon, bone and dentin and of type-III collagen is about one residue per α -chain, and that of type-II collagen from cartilage about two residues per α -chain (Miller & Lunde, 1973; Epstein & Munderloh, 1975; Fietzek & Kühn, 1976; Piez, 1976). On the other hand, the 3-hydroxyproline content of type-I collagen synthesized by cultured fibroblasts during cell attachment was 4–5 times that of normal type-I collagen (Lembach *et al.*, 1977). Type-IV collagens from basement membranes are characterized by a high 3-hydroxyproline content, ranging from about 10 to 15 residues per α -chain (Kefalides, 1973).

The synthesis of the two hydroxyproline isomers is catalysed by separate enzymes, prolyl 4-hydroxylase and prolyl 3-hydroxylase (Risteli *et al.*, 1977; Tryggvason *et al.*, 1977). One possible reason for these differences in 3-hydroxyproline content may lie in differences in the amounts of prolyl 3-hydroxylase activity in various cells. As the 3-hydroxyproline content of the same collagen type can vary (Lembach *et al.*, 1977), it seems possible that prolyl 3-hydroxylase activity may also vary even in the same cell type. Changes in prolyl 4-hydroxylase activity have been studied in a number of animal and human tissues under various experimental and clinical conditions (see Cardinale & Udenfriend, 1974; Kivirikko &

Risteli, 1976; Prockop *et al.*, 1976), whereas possible changes in prolyl 3-hydroxylase activity have been measured only in two studies (Risteli *et al.*, 1978b; Tryggvason *et al.*, 1978). In the present work, attempts were made to gain some information on changes in prolyl 3-hydroxylase activity by comparing its activity in several chick-embryo and rat tissues and by studying age-related changes in its activity in the rat. As differences in prolyl 3-hydroxylase activity may in part reflect differences in the quantity of collagen-producing cells, prolyl 4-hydroxylase activity was assayed in the same samples, and the ratio between the two enzyme activities used for partial correction of this factor.

Experimental

Materials

Male Sprague-Dawley rats fed on a commercial diet (Hankkija Oy, Helsinki, Finland) and allowed free access to water were anaesthetized with diethyl ether and decapitated, and the tissues removed rapidly and immediately frozen in liquid N₂. Twelve newborn rats and four each at ages of 10, 20, 75, and 227 days were used, the tissues from each rat being stored separately except for the age-group 0 days, in which samples of each tissue from three rats at a time were pooled.

The chick embryos were obtained from fertilized eggs of white Leghorn chickens (Siipikarjanhoitajien Litto r.y., Hämeenlinna, Finland) after incubation in a moist atmosphere at 37°C. The tissues were dissected from ten 16-day chick embryos and im-

mediately frozen in liquid nitrogen, two pools being made for each tissue. All samples were stored at -70°C until used.

Preparation of samples for assays

The tissues were thawed and homogenized in a cold (0°C) solution consisting of 0.2M-NaCl, 0.1M-glycine, 1mM- β -mercaptoethanol, 0.1% (w/v) Triton X-100, 0.01% (w/v) soya-bean trypsin inhibitor and 0.05M-Tris/HCl buffer adjusted to pH 7.5 at 4°C .

The liver, kidney-cortex and lung samples from the rats of 0, 10 and 20 days were homogenized in the proportions 1g of tissue/19ml of solution and the skin samples for the same ages in 1g/9ml. The liver, kidney-cortex and lung samples from the 75- and 227-day-old rats were homogenized in the proportions 1g/9ml and the skin samples in 1g/3ml. All tissues from the chick embryos were homogenized in the proportions 1g/9ml.

Skin samples from the rats, and skin, bone and cartilage samples from the chick embryos, were homogenized twice with an Ultra-Turrax homogenizer for 15s each time. Homogenization of the soft tissues was carried out in a Teflon glass homogenizer.

The homogenates were incubated for 30min at 4°C and then centrifuged at 15000g for 30min at 4°C . Portions of the supernatants were used for the assays of the enzyme activities and the supernatant protein. Control experiments with the homogenates of skin and kidney cortex of 10- and 227-day-old rats and of cartilaginous bone and liver of 16-day chick embryos indicated that the conditions used for the extraction of the enzyme activities were optimal and that about 90% of the enzyme activities in the homogenates were recovered in the 15000g supernatants.

Assay of prolyl 3-hydroxylase and 4-hydroxylase activities

For the assay of prolyl 3-hydroxylase activity, portions of the 15000g supernatants were incubated with agitation for 30min at 20°C in a final volume of 1.5ml containing 1000000 d.p.m. of [2,3- ^3H]proline-labelled fully 4-hydroxylated procollagen substrate, 0.08mM- FeSO_4 , 2mM-ascorbic acid, 0.5mM-2-oxoglutarate, 0.2mg of catalase (Sigma Chemical Co., St. Louis, MO, U.S.A.)/ml, 0.1mM-dithiothreitol (Calbiochem, London, W.1, U.K.), 2mg of bovine serum albumin (Sigma)/ml and 50mM-Tris/HCl buffer adjusted to pH 7.8 at 25°C . The reaction was stopped by adding 0.5ml of 10% (w/v) trichloroacetic acid, and the $^3\text{H}_2\text{O}$ formed during the hydroxylation assayed by vacuum distillation of the whole reaction mixture (Risteli *et al.*, 1978a).

For the assay of prolyl 4-hydroxylase activity, portions of the 15000g supernatants were incubated with agitation for 30min at 37°C in a final volume of 2ml containing 60000 d.p.m. of [^{14}C]proline-labelled

procollagen substrate and other compounds as described previously (see Risteli *et al.*, 1976). The reaction was stopped by adding an equal volume of concentrated HCl, and the amount of hydroxy[^{14}C]proline formed assayed after hydrolysis at 120°C overnight (Juva & Prockop, 1966).

Other assays

The protein content of the 15000g supernatant of the tissue homogenates was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

All radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 35% and a background of 10 c.p.m. for ^3H radioactivity, and 85% and 25 c.p.m. for ^{14}C radioactivity. In the assay of prolyl 3-hydroxylase activity, a 1.6ml sample of the $^3\text{H}_2\text{O}$ was dissolved in 5ml of Lumagel (Lumac Systems A.G., Basel, Switzerland) and counted for radioactivity. In the assay of prolyl 4-hydroxylase activity the scintillant reported in the procedure was used (Juva & Prockop, 1966).

Results

Age-related changes in prolyl 3-hydroxylase and 4-hydroxylase activities in the rat tissues

Prolyl 3-hydroxylase activity, expressed per mg of extract protein, was much higher in the kidney cortex than in the liver or skin (Fig. 1a). The enzyme activity in the lung, which was assayed only at the age of 75 days, was 1.3 times as high as that in the liver and twice as high as that in the skin, but less than one-third of that in the kidney cortex. There was a marked decrease in prolyl 3-hydroxylase activity in all tissues beyond 10 days of age, the value at 227 days being about 25% of that on day 10 in the kidney cortex, 13% in the skin and 30% in the liver.

Prolyl 4-hydroxylase activity differed from the above in that the highest values were found in the skin and lung and the lowest ones in the liver (Fig. 1b). This enzyme activity likewise decreased with age in all the tissues studied.

The ratio of prolyl 3-hydroxylase to 4-hydroxylase activity in the kidney cortex was 13–17 times as high as that in the skin (Fig. 2), and the ratio in the liver was 6–8 times and that in the lung about twice that in the skin. No significant changes were found in this ratio with age (Fig. 2).

Prolyl 3-hydroxylase and 4-hydroxylase activities in the 16-day chick-embryo tissues

Most chick-embryo tissues had a high prolyl 3-hydroxylase activity, with values exceeding 3500 d.p.m./mg of protein in all cases except the liver, kidney cortex and lens (Table 1), but, since the prolyl 4-hydroxylase activity was also quite high, the ratios

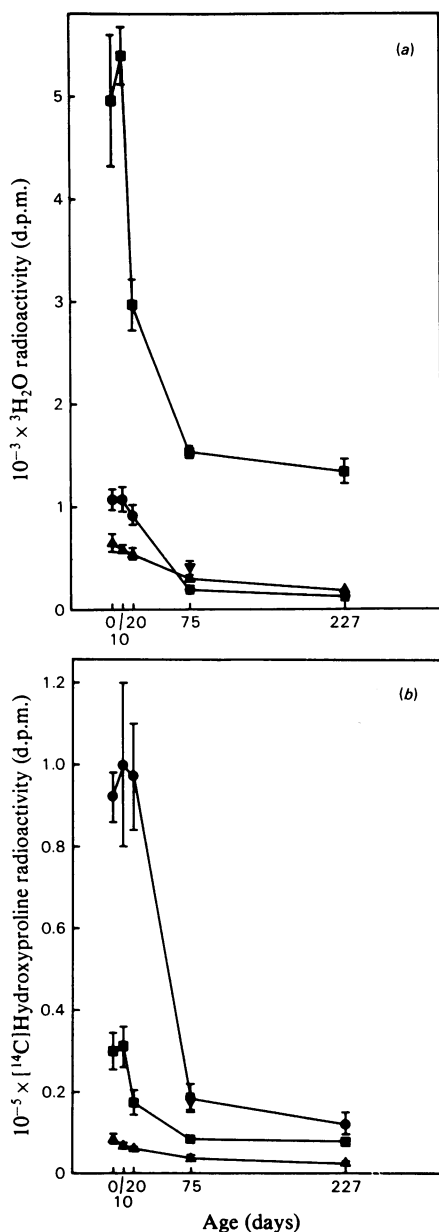


Fig. 1. Age-related changes in prolyl 3-hydroxylase and 4-hydroxylase activities in certain rat tissues

The values are expressed as radioactivity (d.p.m.) of product formed/mg of protein in the 15000g supernatants of the tissue homogenates. The values are means \pm s.d. of four determinations (shown by bars). (a) Prolyl 3-hydroxylase; (b) prolyl 4-hydroxylase. ■, Kidney cortex; ▲, liver; ●, skin; ▼, lung.

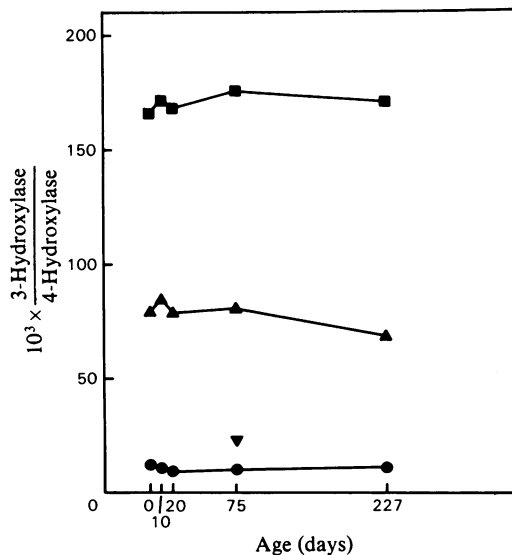


Fig. 2. Ratio of prolyl 3-hydroxylase to 4-hydroxylase activity as a function of age in certain rat tissues

The values are expressed as ratios of the mean values of enzyme activities at each age. ■, Kidney cortex; ▲, liver; ●, skin; ▼, lung.

noted in the rat tissues. Exact comparison between the chick-embryo and rat tissues is not possible, however, as separate substrate preparations were used for the two species. The highest ratios were found in the liver, heart, lens, aorta and kidney, and the lowest in tendon, cartilage, cartilaginous and membranous bone and skin.

Discussion

Changes in prolyl 4-hydroxylase, lysyl hydroxylase and the two hydroxyllysyl glycosyltransferase activities have been studied in many reports during recent years (see Kivirikko & Risteli, 1976; Prockop *et al.*, 1976). It has been found that the activities of these four intracellular enzymes of collagen biosynthesis do not always behave identically, the prolyl hydroxylase and lysyl hydroxylase activities showing considerably larger changes than those of the hydroxyllysyl glycosyltransferases both with age (Uitto *et al.*, 1969; Anttinen *et al.*, 1973; Risteli & Kivirikko, 1976; Anttinen *et al.*, 1977; Tuderman & Kivirikko, 1977) and in experimental hepatic injury (Risteli & Kivirikko, 1974, 1976). The changes in prolyl 3-hydroxylase activity in experimental hepatic injury were found to resemble those in the hydroxyllysyl glycosyltransferase activities in that a much smaller increase was noted than in prolyl 4-hydroxylase activity (Risteli *et al.*, 1978b). The present study

of prolyl 3-hydroxylase to 4-hydroxylase activity were of about the same order of magnitude as those

Table 1. *Prolyl 3-hydroxylase and 4-hydroxylase activities in certain tissues on day 16 of chick-embryo development*
Results are expressed as means \pm deviation for values of two tissue pools each assayed in duplicate.

Tissue	3-Hydroxylase (d.p.m./mg of protein)	4-Hydroxylase [$10^{-3} \times$ (d.p.m./mg of protein)]	$10^3 \times \frac{3\text{-Hydroxylase}}{4\text{-Hydroxylase}}$
Sternum	5090*	280*	18.2
Tibia	5240*	252*	20.8
Femur	5760*	242*	23.8
Cranium	4270 \pm 80	143 \pm 10	29.8
Tendon	6910 \pm 710	458 \pm 23	15.1
Skin	3600 \pm 130	109 \pm 3	33.1
Aorta	5480 \pm 310	103 \pm 7	53.2
Heart	3930 \pm 870	63.5 \pm 4.0	61.8
Liver (metanephros)	1710 \pm 420	25.5 \pm 4.2	67.0
Kidney	1770 \pm 200	38.5 \pm 2.3	46.0
Lens	630 \pm 60	11.5*	55.0

* Duplicate determination of one tissue pool.

indicates that age-related changes in prolyl 3-hydroxylase activity in rat tissues are similar to those in prolyl 4-hydroxylase activity, and thus differ from those in the hydroxyllysyl glycosyltransferase activities.

Considerable differences were found between tissues in the ratio of prolyl 3-hydroxylase to 4-hydroxylase activity. Basement-membrane collagens are rich in 3-hydroxyproline (see Kefalides, 1973), and thus it is not surprising that the ratio was very high in rat kidney cortex, as most of the collagen in this tissue is type-IV collagen (Man & Adams, 1975). Quite high ratios were also found in the liver, but it is not known whether this reflects the synthesis of large amounts of type-IV collagen or an unusually high degree of modification of types-I and -III collagens. Lysyl residues in types-I and -III collagens from the liver are hydroxylated to a greater extent than are those in the skin (Epstein & Munderloh, 1975; Seyer *et al.*, 1977), but the extent of 3-hydroxylation of prolyl residues has not been reported. The ratios in various tissues of the chick embryo can likewise be explained mainly by differences in the synthesis of type-IV collagen or in the extent of prolyl 3-hydroxylation of types-I and -III collagens. Somewhat surprisingly, however, the ratio in the kidney was not very high, and was distinctly lower than in the liver.

The present results, in particular the high prolyl 3-hydroxylase activity in the rat kidney cortex, suggest that differences in the extent of prolyl 3-hydroxylation between various collagens can in part be explained by differences in the amount of prolyl 3-hydroxylase activity between different cells, but this is apparently not the only reason. The time required for triple-helix formation in the biosynthesis of procollagen varies considerably between cells synthesizing different types of collagen (see Grant & Jackson, 1976; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976). Prolyl 3-hydroxylase does not catalyse

its reaction with a triple-helical substrate (Risteli *et al.*, 1977), and it thus seems probable that the time available before triple-helix formation in the cell may be partly responsible for the differences in 3-hydroxyproline content between the different collagens. Interestingly, a decreased 3-hydroxyproline content has been found in the glomerular basement-membrane collagen of patients with congenital nephrotic syndrome of the Finnish type (Tryggvason, 1977; Tryggvason *et al.*, 1978), even though no decrease in prolyl 3-hydroxylase activity in the kidney cortex was noted (Tryggvason *et al.*, 1978).

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